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Electrochemical Molecular Beacon for Nucleic Acid Sensing in a Homogeneous Solution

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Ferrocene (Fc) and β-cyclodextrin (βCyD) were modified at each end of stem-loop structured DNA as an electrochemical signal generator and its quencher, respectively, to give an electrochemical molecular beacon (**eMB**). A relatively high efficiency of signal quenching was achieved by an inclusion complex (βCyD $\supset$ Fc) formation that was induced on the stem structure of the closed form (= stem-loop structure) of **eMB**. With the addition of target DNA, the structure of **eMB** opened to form a linear duplex, where the Fc dissociated from the βCyD to restore its intrinsic electrochemical signal. The signal contrast of the electric current for this off/on-type sensor was high, *ca*. 95. This technique did not require any modification of the electrode surface, and it realized the detection of the target nucleic acids in a homogeneous solution with a high sensitivity using high-performance liquid chromatography (HPLC) equipped with electrochemical detector.

Keywords Nucleic acids, electrochemical biosensor, molecular beacon, inclusion complex, ferrocene, cyclodextrin, homogeneous assay

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Introduction

Various fluorescent molecules or nano-materials have been developed as probes for monitoring targeted biomolecules using various forms of spectroscopy. In most cases, these probes are required to provide a signal in a spatiotemporal manner. That is, they are designed to generate a signal by specific bio-chemical or -physical stimuli that are relevant to the biological events of interest.[1] Molecular beacon (MB) is a typical stimulus-responsive fluorescent molecular probe consisting of an oligodeoxyribonucleotide (ODN) and a FRET (Förster resonance energy transfer) pair. The fluorescence from a dye attached to one end of the ODN is quenched with the acceptor attached on another end by FRET in the hairpin-form (stem-loop) structure of the MB. The interaction with the target DNAs or RNAs changes the conformation of the MB to a rigid linear duplex to break up the FRET pairing, and then the intrinsic fluorescence of the dye is restored.[2] MBs have been widely used as general signal transducers for various biomolecules, even those other than DNA or RNA, such as small molecules, proteins with a combined use of aptamers for their targets.[3]

Electrochemical techniques are cost-effective and potentially sensitive and versatile. Although various analytical platforms based on electrochemical responses have been proposed for studying specific interactions involving nucleic acids and/or proteins, the number of applications used in practical assays is quite limited compared with fluorescent techniques. This is mostly due to the lack of a means for modulating of an electrochemical signal in a homogeneous solution. Most electrochemical methods, therefore, require one of the participants in the interactions to be immobilized onto the electrode, because signal contrasts arise as a result of changes in the distance between the electrochemically active probe and the electrode surface on the targeted events.[4] The preparation of biomolecule-modified electrodes is time-consuming and, generally, it causes difficulty in obtaining reproducible results using two electrodes prepared even with the same protocol. If molecular quenchers of electrochemical signals are available, electrochemistry-based MBs (**eMB**s) could be designed and electrochemical monitoring for biological events could be performed in a homogeneous solution.

Previously, we proposed electrochemical DNA sensing using a ferrocene (Fc)-modified DNA probe.[4a–4c,5] In that series of studies, we found that a β-cyclodextrin (βCyD) modified on the end of an ODN almost completely suppressed the electrochemical signal from the Fc. The βCyD and Fc tethered to the ends of different ODNs form a tight inclusion complex on the DNA scaffolding (single-stranded target DNA). The Fc in the cavity of the βCyD is shielded from the bulk solution, so its electron transfer with the electrode is suppressed. In this case, the βCyD is therefore a good quencher for electrochemical signals, similar to an azo-quencher used in FRET-based conventional MBs.[5] This means that the **eMB** can be constructed based on an appropriate hairpin form of an ODN by tethering the Fc and βCyD on each end, respectively (Fig. 1). **eMB** works in a homogeneous solution and frees the electrochemical sensing of the targets from the need to anchor the sensor molecules on the electrodes.

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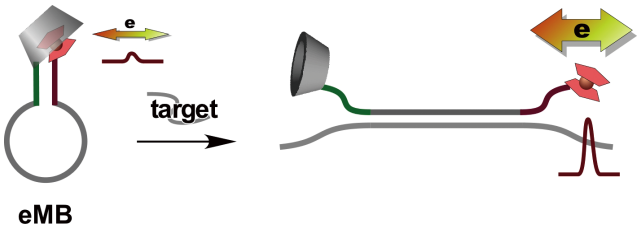


Fig. 1 Schematic illustration of electrochemical molecular beacon (**eMB**).

Experimental

General

The Fc carboxylic acid and *O*-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium tetrafluoroborate (TBTU) were purchased from Tokyo Chemical Industry (Tokyo, Japan). 1-Hydroxybenzotriazole monohydrate (HOBt) was purchased from Watanabe Chemical Industries (Hiroshima, Japan). The βCyD and *N*-succinimidyl 3-(2-pyridyldithio) propionate (SPDP) were purchased from Sigma-Aldrich (Saint Louis, MO, USA) and Dojindo Laboratories (Kumamoto, Japan), respectively. All ODNs were synthesized by an automated DNA synthesizer (8900, Expedite or NTS M-2-MX, Nihon Techno Service) using conventional phosphoramidite methods. The phosphoramidite monomers were purchased from Proligo (Hamburg, Germany) and Glen Research (Sterling, VA, USA). All ODNs and ODN conjugates were purified by reversed-phase HPLC (Column: InertSustain Swift C18, 4.6 mm $\varphi \times 150$ mm, GL Science, Tokyo) using an appropriate linear gradient with 0.1 M triethylamine-acetic acid (TEAA)-acetonitrile (pH 7.0), and identified by MALDI-TOF mass spectrometry on a Bruker Daltonics Autoflex-III (Billerica, MA, USA) using 3-hydroxypicolinic acid as a matrix. All other reagents were obtained as the highest grade and used without further purification.

*Preparation of the **eMB***

An outline of the total syntheses of the **eMB** is shown in Scheme 1. The **eMB** was synthesized by two successive post-synthetic modifications of the Fc and βCyD with the alkyl amino groups modified on each end of an ODN. The first coupling of the Fc carboxylic acid was performed with the 5′-aminated ODN tethered to the support resin (CPG, controlled pore glass). Afterward, βCyD modification at the 3′-end of an ODN was carried out in a solution after cleavage from the CPG.

Fc-ODN: Prior to coupling, CPG (0.4 mg, carrying 15 nmol ODN as expected) was treated with 500 µL of 10% triethylamine in acetonitrile for 10 min, and the solution was removed from the CPG after settling. The Fc carboxylic acid (3.5 mg, 15 µmol), HOBt (2.0 mg, 15 µmol) and TBTU (4.8 mg, 15 µmol) were dissolved in a 100-µL mixed solution of acetonitrile and *N*,*N*-dimethylformamide (DMF) (7:3) to prepare the Fc solution. To 20 µL of the Fc solution, CPG was added and incubated for 1 h at room temperature with gentle agitation. Another 20 µL of a fresh Fc solution was added to the CPG suspension and mixed overnight at room temperature. The CPG suspension was allowed to settle and the solution was removed. Then, the CPG was washed with acetonitrile. The ODNs were cleaved from the CPG by methylamine and the **Fc-ODN** was purified with reversed-phase HPLC and identified with MALDI-TOF MS according to the standard protocols.[6] MALDI-TOF MS: *m*/*z* calcd for $[M–H]^-$: 9667.1; found: 9666.6.

Fc-ODN-ssPy: The **Fc-ODN** (20 nmol) was dissolved in 40 µL of 10 mM phosphate buffer (pH 9.0, containing 10 mM EDTA). To this solution, SPDP (0.6 mg, 2.0 µmol) dissolved in 20 µL of dry DMSO was added. The resulting suspension was shaken for 20 min at room temperature and subjected to a NAP-10 column (Sephadex G-25) to remove excess SPDP. Finally, the **Fc-ODN-ssPy** was isolated with reversed-phase HPLC and identified with MALDI-TOF MS according to the standard protocols. MALDI-TOF MS: *m*/*z* calcd for $[M–H]^-$: 9864.4; found: 9868.1.

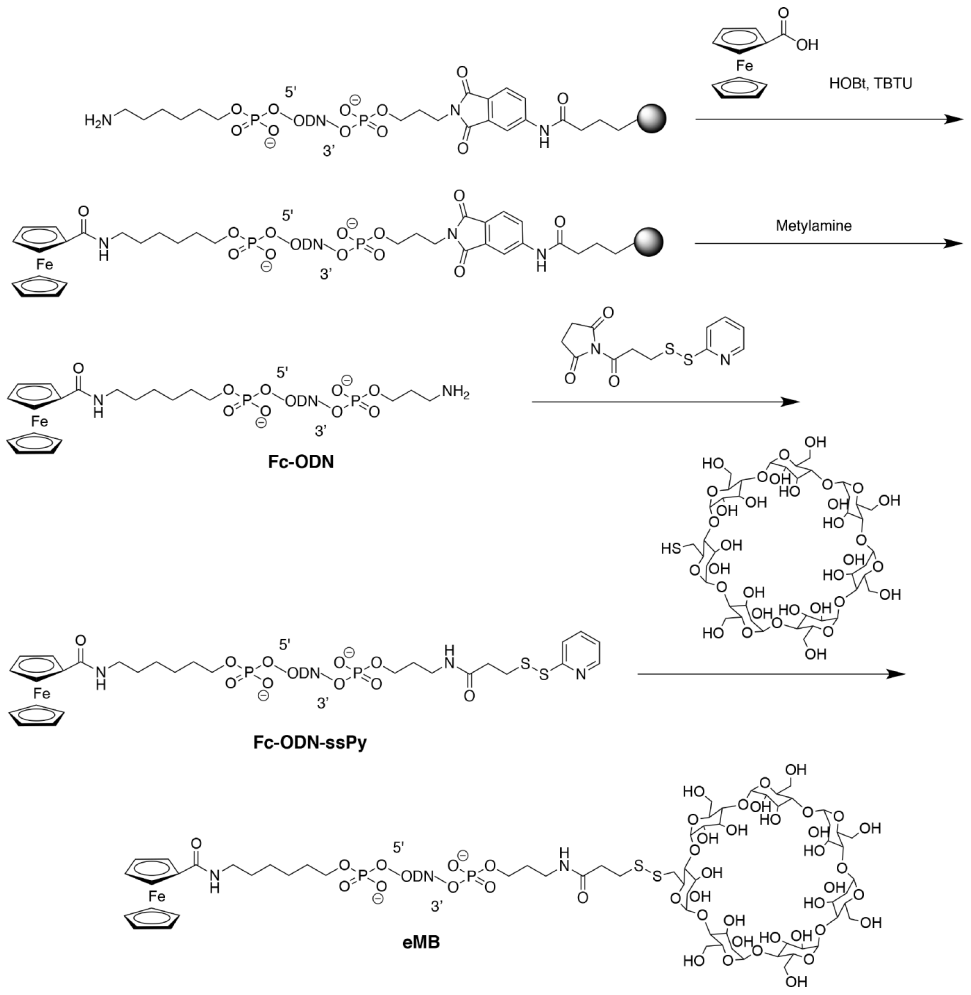
eMB: Thiolated βCyD was prepared *via* tosylated βCyD according to a procedure previously reported.[5] The **Fc-DNA-ssPy** (11 nmol) was dissolved in 20 µL of 10 mM phosphate buffer (pH 7.0, containing 10 mM EDTA). To this solution was added thiolated βCyD (100 nmol) dissolved in 10 µL dry DMSO. The resulting suspension was stirred at room temperature overnight. The solution was subjected to reversed-phase HPLC to purify the **eMB** using a standard protocol. The **eMB** was identified with MALDI-TOF MS. MALDI-TOF MS: *m*/*z* calcd for $[M–H]^-$: 10904.3; found: 10964.4.

UV melting experiments

Thermal denaturation experiments were carried out in a Tris–HCl buffer solution (20 mM, pH 8.5) containing 1.0 M NaCl. The concentration of each DNA component was 1 µM. All experiments were performed on a UV/Vis spectrophotometer equipped with a Peltier thermal controller (1650pc, Shimadzu). Prior to beginning each denaturation experiment, the samples were degassed at 85°C for 5 min, and then annealed with slow cooling to 0°C. After equilibration for 30 min at 0°C, the solutions were heated slowly at a rate of 0.5°C min^{-1} while blow drying the cuvette with N_2 gas (below room temperature) to prevent condensation. The melting profiles were monitored by an absorption change at 260 nm. The temperature that gave the maximum first derivative of the melting curve was used as the melting temperature (T_m), an index of the thermal stability of the structures. Thermal denaturation experiments were repeated two to three times for each sample and the reported T_m values were the average of all duplicates or triplicates for a given sample.

Electrochemical measurements

Differential pulse voltammetry. Electrochemical measurements were performed using an Electrochemical Analyzer (ALS 842B, BAS) with a conventional three-electrode system at room temperature in 10 mM phosphate buffer (pH 7.0) containing 500 mM KCl. A glassy carbon disk (1.0 mm φ), platinum wire, and Ag/AgCl (with 3.0 M NaCl) were used as the working, auxiliary, and reference electrodes, respectively. Differential pulse voltammetry (DPV) was performed with scanning from +0.2 to +0.6 V with a 200-ms pulse period, a 25-mV pulse amplitude, a 50-ms pulse width, and a 4-mV potential increment. *Electrochemistry in the flow system*. Quantitative electrochemical measurements using the **eMB** were performed with the HPLC equipped with an electrochemical detector (ECD) (ECD-700, Eicom). After the addition of targets to **eMB**, sample solutions were incubated for 15 min at room temperature prior to measurements. Ion-exchange chromatography (TSKgel DNA-STAT, 4.6 mm $\varphi \times 100$ mm, Tosoh) was adopted for determining DNAs with a linear gradient using 0.02 M Tris–HCl (pH 8.5) (Eluent A) and 0.02 M Tris–HCl with 2.0 M NaCl (pH 8.5) (Eluent B) as the eluents (gradient, 10 – 50% B in 30 min; flow rate, 0.6 mL/min). The chromatograms were recorded at several applied potentials using the ECD with a glassy carbon electrode. A hydrodynamic voltammogram of **Fc-ODN** was obtained from plots of the current peaks at several potentials for the same amount of **eMB** (200 pmol).



Scheme 1 Synthesis of **eMB**.

Results and Discussion

The sequences and the structure of the **eMB** and other ODNs used in this study are shown in Fig. 2. The 5′- and 3′-ends of the ODN were modified with the Fc and βCyD, respectively, to prepare the **eMB**. The length of the linker chains tethering both ends of the ODN were designed to be almost the same, so a stable inclusion complex, βCyD $\supset$ Fc, would form at the end of its hairpin conformation. The **eMB** was obtained by two-step post modifications of the ODN with amino alkyl linkers on both ends. To conduct each successive modification selectively, the Fc carboxylic acid was attached to the 5′-end amino group of the ODN on the CPG by coupling using TBTU/HOBt. Then, monothiolated βCyD was modified to the 3′-end of the **Fc-ODN** after liberation from the CPG through a bifunctional linker, SPDP. The yield of the Fc coupling to the ODN on the CPG was very low, typically around 20 – 30% (Fig. S1, Supporting Information). Although we tried to improve the yield by modifying various conditions, such as the CPG pretreatment method, the kinds of coupling reagents, the quantity of chemical ingredients, the reaction times and the temperatures, we could not find remarkable solutions. We finally adopted the amide bond formation on the support resin using TBTU/HOBt, which is one of the most common reactions in peptide synthesis. Surface structures of the CPG used in DNA synthesis might not be ideal for such coupling reactions to form an amide bond. Other reactions were conducted as described in the literature,[7] in some cases, with minor modifications. Their yields were reasonable (Figs. S2 and S3, SI).

Melting experiments were carried out for the **eMB** in the absence and presence of two ODNs, $\mathbf{T_L}$ and $\mathbf{T_S}$, each of which consisted of a 20-mer sequence complementary to the central loop and a partial loop plus a stem at the βCyD side of the **eMB**, respectively. The **eMB**, itself, gave a gentle transition at around 38°C, which was derived from a conformational change from the hairpin to the coil (Fig. S4a, SI). However, relatively sharp transitions were observed for the duplexes formed between the **eMB** and $\mathbf{T_L}$ or $\mathbf{T_S}$ (**eMB/**$\mathbf{T_L}$ and **eMB/**$\mathbf{T_S}$) at *ca*. 65 and 71°C, respectively (Fig. S4b). Generally, the steepness of the melting curve reflects the degree of entropy change; the steeper the melting is, the more $-\Delta S$ it shows.[8] The gentle and sharp transitions observed for **eMB** and its duplexes (**eMB/**$\mathbf{T_L}$ and **eMB/**$\mathbf{T_S}$) agree with their intramolecular and intermolecular transitions, respectively. Considering the T_m values, the open form (duplex) of the **eMB**s (**eMB/**$\mathbf{T_L}$ and **eMB/**$\mathbf{T_S}$) were thermodynamically more stable than the closed form (hairpin) of the **eMB**.

The fundamental electrochemical property was studied by DPV measurements. As shown in Fig. 3, an obvious peak derived from the oxidation of the Fc was observed at +0.40 V (*vs*. Ag/AgCl) for the **Fc-ODN**. However, the signal of the **eMB** was *ca*. 90% smaller than that of the **Fc-ODN**. This implied that an inclusion complex of βCyD $\supset$ Fc formed in the

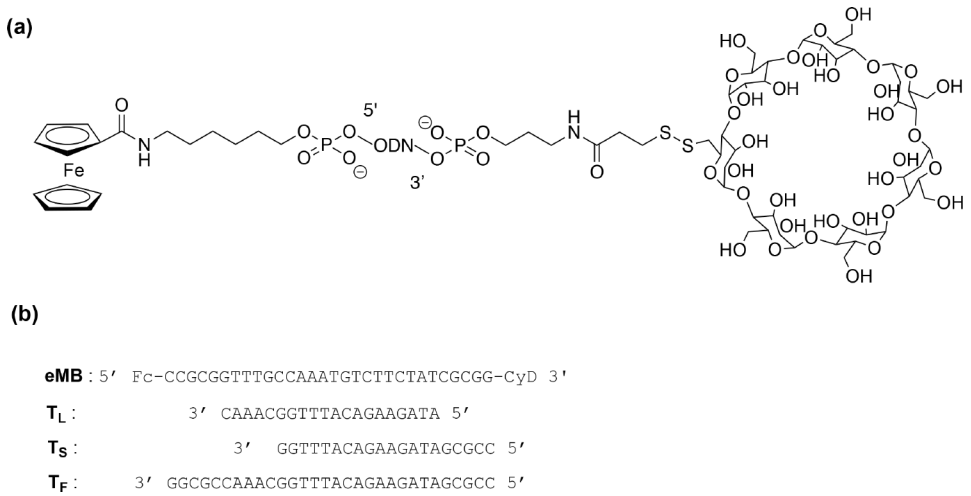


Fig. 2 (a) Structure of **eMB** and (b) the sequence of DNAs used in this study.

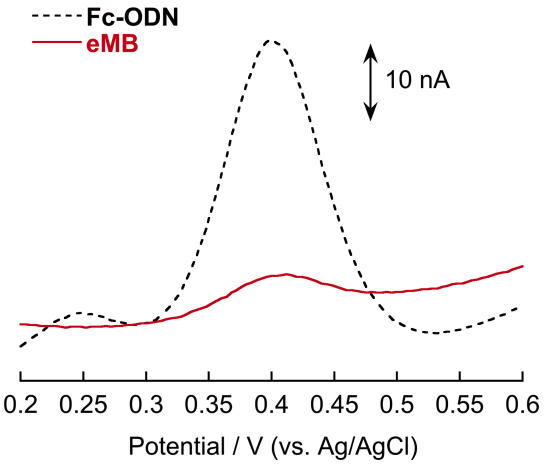


Fig. 3 Differential pulse voltammograms of **Fc-ODN** (black dashed curve) and **eMB** (red solid curve). Measurements were carried out in 10 mM phosphate buffer (pH 7.0) containing 500 mM KCl at 25°C with the scanning potential from +0.2 to +0.6 V with 200-ms pulse period, 25-mV pulse amplitude and 50-ms pulse width, 4-mV potential increment. Concentrations of **Fc-ODN** or **eMB** were 40 μM.

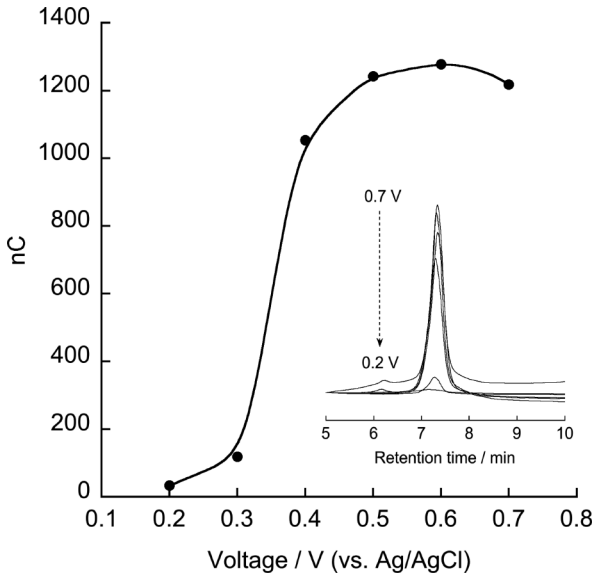


Fig. 4 Hydrodynamic voltammogram of **Fc-ODN** measured by HPLC equipped with EDC. The inset shows chromatograms of **Fc-ODN**s at several applied potentials. Column: TSKgel DNA-STAT, 4.6 mm $\varphi \times 100$ mm, Tosoh; eluent A: 0.02 M Tris–HCl (pH 8.5); eluent B: 0.02 M Tris–HCl with 2.0 M NaCl (pH 8.5); gradient: 10 – 50% B in 30 min; flow rate: 0.6 mL/min. Amount of **Fc-ODN**s injected were all 200 pmol.

closed hairpin form of the **eMB** to shield the Fc from outside, resulting in an effective suppression of its electron transfer.

The electrochemical signal from the **eMB** was also monitored on the HPLC equipped with an ECD. At the beginning, optimization of the voltage applied to the electrochemical cell was carried out by increasing it from +0.2 to +0.7 V (*vs.* Ag/AgCl) using the **Fc-ODN**. The oxidation peak of the Fc was found at around 7.2 min on the chromatogram (Fig. 4 inset). The peak currents were increased by raising the voltage, which reached a plateau at +0.5 V, a little higher than the oxidation potential of the Fc.[10] Thus, the voltage applied on the ECD in following experiments was set at +0.5 V (Fig. 4).

In general, the detection limit of MBs based on the fluorescent signal depends on a T_m value of the duplex formed between the MB and the target, which determines the amount of open form of the MB and, accordingly, the amount of signal. Although the T_m value is essential for the detection limit of this system, it can be controlled by the length of the complementary sequence of the MB with the target and salt concentration. Therefore, the detection limit of this system is also expected to change according to the length of target and given experimental conditions, and can be controlled by the probe design and buffer conditions, like that of a conventional MB. Therefore, we tried to find the potential (theoretical) detection limit of this system, which is simply affected by the amount of the Fc, rather than the amount of the open form of the **eMB**. For these reasons, the peak currents were measured by changing the amount of the **Fc-ODN** to assess the potential detection limit of this system. As shown in Fig. 5, a linear relationship was confirmed between the amount of **Fc-ODN**s and peak currents in the range from 1 to 100 pmol. Even only 500 fmol of the **Fc-ODN** was clearly

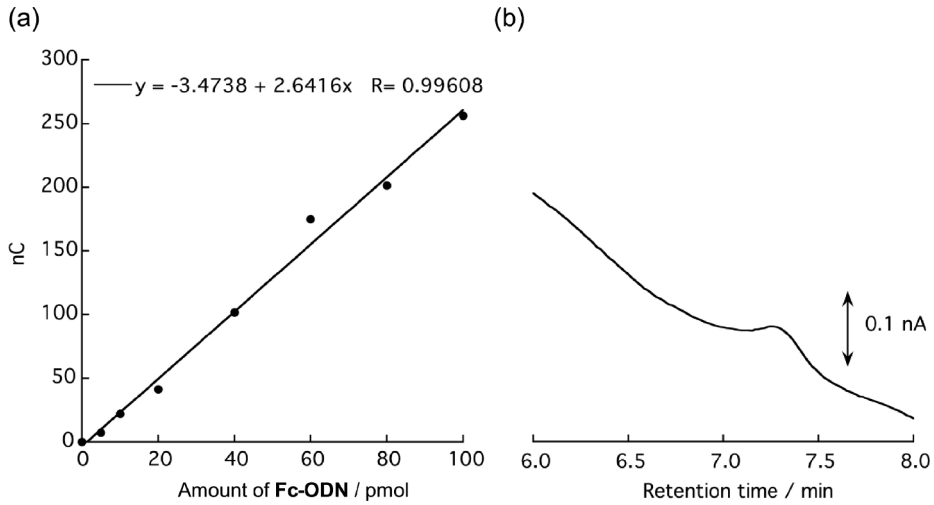


Fig. 5 (a) Amount of **Fc-ODN** *vs.* current plot measured by HPLC equipped with EDC in the range from 1 to 100 pmol. (b) HPLC chromatogram for the detection of 500 fmol **Fc-ODN**.

detected as an obvious peak. This implies that some amount of the target DNA that opens 500 fmol of the **eMB** can be detected in this system.

The chromatograms obtained by HPLC-ECD are shown in Fig. 6. Contrary to our expectation, the electrochemical response of the **eMB** was hardly restored by the addition of $\mathbf{T_L}$. Scarce signals observed at 7.4 and 8.4 min would be attributed to the closed forms of the **eMB** and **eMB/T$_L$**, respectively. Although it is inconsistent with the result of the UV melting experiment with $\mathbf{T_L}$, we thought that some unknown factors under the conditions of the ion-exchange mode in HPLC destabilized the **eMB/T$_L$** and/or promoted the formation of more complicated DNA complexes. Complementarity of 5-nucleotides-long stem sequences on both ends and flanking the Fc and βCyD of the **eMB** or **eMB/T$_L$** might be responsible for the discrepancy. Thus, similar experiments of chromatography were conducted using $\mathbf{T_F}$, which has a fully complementary sequence of **eMB**, instead of $\mathbf{T_L}$. Remarkable restoration of the electrochemical signal was observed for $\mathbf{T_F}$ (Fig. S5), probably due to the masking of the 5-nuleotide sequences in the stem region of the **eMB**. However, the generality of the probe design is lost if it is necessary for the targets to be complementary to not only the loop, but also to both stem sequences of the **eMB**. To ensure the generality, we used $\mathbf{T_S}$ as a model target, which is complementary to the loop and one stem region of the **eMB**. This design of the probe did not restrict the target sequence. By the addition of $\mathbf{T_S}$, a large recovery of the current signal was observed, as shown in Fig. 6; the signal ratio in the absence and presence of $\mathbf{T_S}$ (on/off ratio) was *ca.* 95. Previously, Aoki *et al.* reported an electrochemical DNA probe modified with a Fc and βCyD. Although it showed a *ca.* 60 mV shift in its oxidation potential when binding with the target sequence, the on/off ratio of the electric current was *ca.* 5 at an arbitrary potential.[11] The binding constant between the Fc and βCyD was not high (K = 1.65×10^4 M^{-1}). Therefore, it seems that the signal suppression in the absence of the targets was sacrificed, resulting in a low signal contrast. However, that probe design, which did not involve a stem region, was simple and versatile. The results of the present study indicate that the short sequence of the stem moiety is essential when designing an **eMB** with a high on/off ratio in its electric current. The stem structure is important not only for the complete quenching of the electrochemical signal

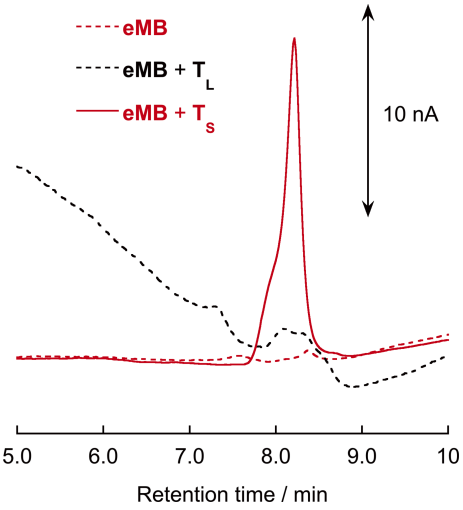


Fig. 6 Chromatograms of **eMB** in the absence (red dashed curve) and presence of $\mathbf{T_L}$ (black dashed curve) or $\mathbf{T_S}$ (red solid curve). Column: TSKgel DNA-STAT, 4.6 mm $\varphi \times 100$ mm, Tosoh; eluent A: 0.02 M Tris-HCl (pH 8.5); eluent B: 0.02 M Tris-HCl with 2.0 M NaCl (pH 8.5); gradient: 10 – 50% B in 30 min; flow rate: 0.6 mL/min. Amount of **eMB**s and $\mathbf{T_L}$ or $\mathbf{T_S}$ injected were all 100 pmol.

by the placing auxiliary units in close proximity using stem hybridization as a guide, but also for avoiding a false opening of the MB. The stem-induced hairpin form of the MB is a metastable structure that competes with the duplex formed with the target strand, making the sequence recognition of the probe very strict. To the best of our knowledge, this **eMB** is the first molecular beacon consisting of a conventional stem-loop structure based on electrochemical signaling.

Conclusions

We proposed a molecular beacon based on an electrochemical signal induced by a single-stranded target DNA or RNA. The **eMB** consists of a stem-loop DNA, an electrochemical signal

generator (Fc), and its quencher (βCyD), which is similar to the fluorescence-based conventional MB. This technique did not require any modification of the electrode surface essential for a traditional electrochemical biosensor and it realized the detection of target nucleic acids in a homogeneous solution. In addition to the intrinsic high sensitivity of the electrochemical detection system, a relatively high quenching efficiency of the signal was achieved by the DNA-templated formation of an inclusion complex (βCyD $\supset$ Fc) at the terminus of the stem moiety to give a high on/off ratio between the absence and presence of the target DNA. Owing to the simplicity and generality of the probe design, it can become a universal electrochemical sensor for several biomolecules other than the nucleic acids combined with DNA/RNA aptamers. In addition, as with a fluorescence-based multi-color assay, it would be possible to achieve a multi-target assay by the simultaneous use of several electrochemical active molecules that have different redox potentials from each other and can form inclusion complexes with CyD.

Acknowledgements

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Supporting Information

RP-HPLC chromatograms for the isolation of the **Fc-ODN**, **Fc-ODN-ssPy2**, and **eMB** after each synthesis. UV melting curves for the **eMB**, **eMB/T$_L$**, and **eMB/T$_S$**. Chromatograms of the **eMB** in the absence and presence of **T$_F$**. This material is available free of charge on the Web at http://www.jsac.or.jp/analsci/.

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